

## Tissue arrays as fiducial markers for section alignment in 3-D reconstruction technology

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### Abstract

Combination of conventional histology and the three-dimensional spatial view of tissue structures offers new prospects for understanding and diagnosing nature and development of human diseases. The essential technical problem related to three-dimensional reconstruction in histopathology is represented by the correct alignment of serial sections. During the past years several methods have been proposed but failed to become popular because of their limits in terms of time consume and restricted applicability. We aimed to overcome this problem by applying the technology of Tissue Array, thus by positioning adequate fiducial markers from specific "donor" blocks into the "recipient" paraffin block of interest. Digitized pictures of serially cut sections were aligned according to the tissue markers embedded by Tissue Array, and then processed with specific softwares for three-dimensional reconstruction. Thirteen models, including fetal hearts, breast and thyroid carcinomas, were elaborated. We found the procedure to be easy, fast and reproducible. Moreover, by selectively embedding the fiducial markers according to specific angles, the Tissue Arrays can be exploited in order to establish the distance between sections. This original methodology of incorporating Tissue Arrays into paraffin blocks as fiducial markers for three-dimensional reconstruction has a potential impact on histology for research purposes and diagnostic applications.

**Keywords:** Tissue Array • marker • alignment • 3-D reconstruction

### Introduction

Spatial organization is an essential parameter in order to understand the significance of biological structures and the development of biological and pathological processes. Since the very beginning of histological sciences, reconstruction of serially-sectioned tissues has been pursued, using time-consuming procedures, based on cut-out wax plates [1] or cardboard models [2, 3]. In more recent times, computer generated images of digitalized pictures [4], and dedicated softwares offered an easier approach.

Still, technical problems are preventing three-dimensional (3-D) reconstruction from gaining a more extensive popularity in rendering and practice in histological studies of normal and pathological tissues. It is commonly believed that a (if not the) major problem is the alignment of serial two-dimensional sections in a correct sequence in order to build up a 3-D structure. A correct alignment has to stand on reference points, and several methods have been proposed in order to introduce such landmarks inside or near to the sections.

Dorup [5] employed cubic tissue blocks, so that the corners of the sections were used as reference. This solution is of course only feasible in very special occasions and tissues. Johnson and coworkers [6]

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suggested the introduction of prolene strands, a commonly used surgical material, placed around the resin-embedded tissues and working as external fiducial markers. However, prolene can only be cut with diamond knives, and this interesting approach could not be applied to paraffin-embedded tissues. Other authors afforded the problem of introducing fiducial markers into paraffin-embedded tissues by drilling holes using either an ophthalmological laser beam [7] or a jeweller's drill [8]. Such 0,3 mm diameter holes, were either left empty on the tissue block or, as proposed by Sundsten and Prothero [9], filled with fixed peripheral nerves, which on the cut sections appeared as a roundish, easily recognised landmark foreign to the tissue to be studied.

None of these processes, either too cumbersome or impractical, gained widespread popularity, and the placement of external fiducial markers, represented by drilled holes, has only been practiced on resin embedded material [10, 11]. In fact, only resin could guarantee the rigidity of the sections required in order to maintain the objective value of the constructed reference.

We are here proposing a new approach to the positioning of fiducial markers (either external or internal to the tissue block) by exploiting the recently introduced technology of Tissue Arrays. Apparatuses devised to extract selected cores from paraffin embedded tissues, which are routinely employed in Histopathology Labs, can be reversibly employed to introduce easily recognizable cylinders functioning as markers around or inside an embedded tissue block. The section congruency characterizing the Tissue Array technology [12] guarantees that the position of the cores functioning as fiducial markers is maintained on the serial sections, a prerequisite for allowing realignment and 3-D reconstruction.

## **Material and methods**

### **Case selection**

Tissue specimens were obtained from surgical or autopsy material, routinely fixed in 4% buffered formaldehyde and embedded in paraffin. As a source of ("donor") tissue arrays to be grafted into selected blocks to be studied, we employed anthracotic lymph nodes, cerebral cortex or skeletal muscle from surgical specimens or

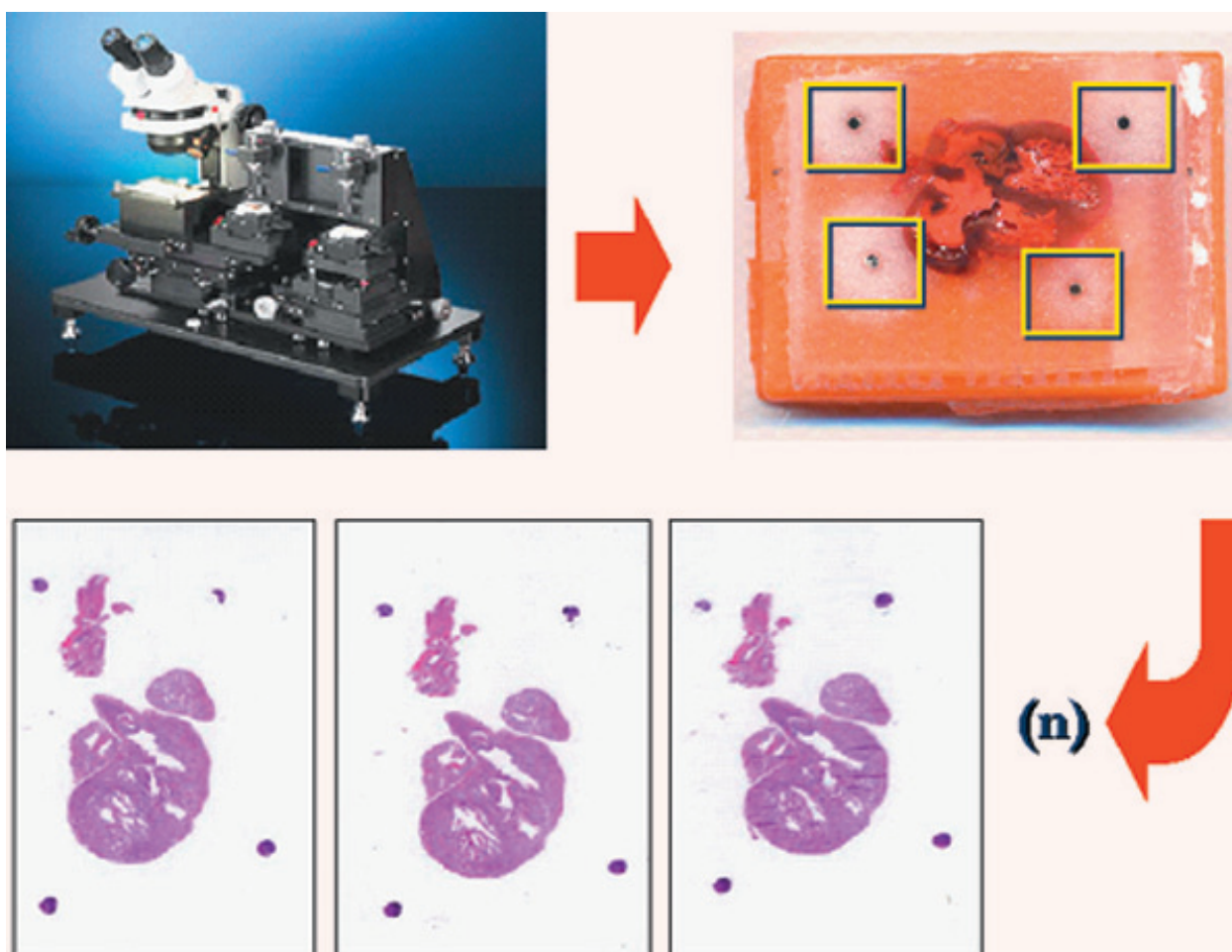
autopsies of patients died for unrelated causes. Such Tissue Arrays were grafted into paraffin blocks of cases of interest for 3D reconstruction, *i.e.* 5 cases of fetal hearts (about 1 cm long) from autopsies of 14 to 23 week old abortions, or surgical material of tumor cases of interest (5 cases of breast cancer and 3 case of thyroid carcinoma).

Fiducial markers positioning by means of Tissue Arrayer. The Advanced Tissue Arrayer (mod. ATA-100, Chemicon International, Tamecula, CA) was used to remove from "donor" blocks cores of tissue selected as reference marker, and to arrange those tissue arrays into "recipient" paraffin blocks, near to the area to be serially cut and 3-D reconstructed. The whole process consists of three subsequent operations.

- 1) Donor blocks (standard plastic cassettes containing paraffin-embedded tissues such as anthracotic lymph nodes or skeletal muscle) were placed into the Donor Block Holder of the instrument. An Haematoxylin and Eosin (H&E) section of the same block was placed on the microscope stage, in order to select the proper area to be punched with a needle on the paraffin block. Needles of different size can be used (from 0,6 mm up to 2 mm) but in our experience the thinnest one was properly fitting to our purpose. The needle with a core of the donor tissue is removed.
- 2) The recipient block containing the tissue of interest for 3-D reconstruction is placed in the Recipient Block Holder, while an H&E stained section of the same is inserted under the microscope. On such section, the proper areas for insertion of the reference markers have been selected in advance. Using the Ocular reticle, such areas are identified on the paraffin block and a needle of appropriate size (usually 0,6 mm) is inserted as to remove a core (later to be discarded) and leave a hole of appropriate size and location.
- 3) The donor needle is set in place and the core reference tissue is delivered precisely inside the hole onto the tissue of interest.

Such process can be repeated a number of times. A schematic illustration of the procedure is represented in Fig. 1.

The recipient blocks with a number of reference markers (from 3 to 5) in place is now ready for sectioning. It is advisable to leave the block on a oven at 37°C for 30', so as to favour the connection of the grafted reference tissue array with the paraffin of the recipient block. Microtome sectioning will then proceed as usual. Reference markers will retain the proper position on the section will easily be



**Fig. 1** Tissue Array procedure to embed fiducial tissue markers in the recipient paraffin block under investigation. Tissue cores from a donor block are introduced at the periphery (or inside) the target tissue (a fetal heart in this example), and remains in the serially cut sections as alignment references

recognised so as to allow alignment of the sections.

Measure of the distance between sections

The interval between sections is a key factor for 3-D reconstruction, especially when the latter is not based on serially cut sequential sections, but on samples collected at a constant interval.

The tissue array approach can help to measure the interval between sections by using, as an internal reference, two arrays at different angles.

In close proximity to a perpendicular array (x), a leaning one (y) is introduced by attaching the tissue block to be studied over an inclined holder of a given angle. The distance between sections (t) will be related to the distance, in different sections, between array x and array y, as well as to the angle (a) of the leaning array (see Fig. 2) and will be governed by the formula:

$$t = (a_2 - a_1) \tan \alpha$$

When the angle  $\alpha$  is  $45^\circ$ , the difference between the two measures  $a_2$  and  $a_1$  will correspond to the interval (t) between sections. Selected samples (one fetal heart and one breast carcinoma sample) were used to verify this approach.

The angle  $\alpha$  (see Fig. 3) can also be reckoned by the formula:

$$\alpha = \arcsin (d_1/d_2)$$

Section alignment and 3-D reconstruction. Different tissues were processed following the above sequence. For each tissue sample under investigation, 4 micron thick sections were serially cut and collected on sylane-coated slides. The number and staining of sections varied according to the purpose and modalities selected for 3-D reconstruction. In most cases conventional H&E staining was performed, while in some samples the following addition-

al immunohistochemical markers were chosen: keratin AE1/AE3 for breast (monoclonal antibody, clone AE1-AE3-PCK26, pre-diluted; Diapath, Ventana, Tucson, AZ) to reveal epithelial cells; CD31 for thyroid (monoclonal antibody, clone JC/70A, diluted 1:10; DAKO Glostrup Denmark) to reveal endothelial cells underlining blood vessels. Immunohistochemical reactions were performed in an automated immunostainer (Ventana BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ).

Digital pictures of the stained sections were serially captured at selected magnification with an OLYMPUS® DP11 digital camera mounted on an OLYMPUS® VANOX T microscope allowing for moving and turning on the stage, so that the reference markers were always set in the same position in the pictures. This approach allowed to take pictures from serial sections of the same histological area in a precise, reproducible, easy and relatively fast sequence. The color images were stored in JPG format with high quality compression using ADOBE® Photoshop, to be processed with the selected software for 3-D reconstruction.

Two different 3-D reconstruction softwares were used. As a first step, images were imported in the SURFdriver 3.5 reconstruction program (SURFdriver, Kailua, Hawaii; www.surfdriver.com) and the 3-D reconstruction consisted of:

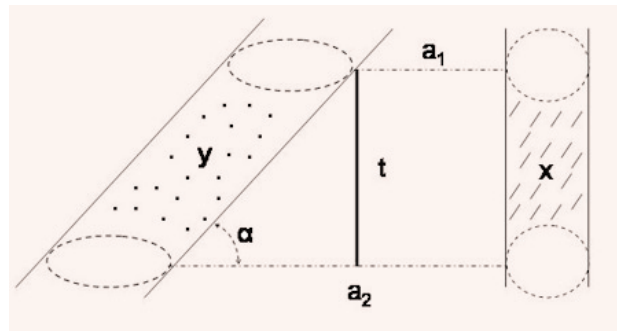
- image segmentation, by manually or semi-automatically underlying the edges of the region of interest
- sections alignment, by overlapping in serial images the reference point corresponding to the fiducial marker from Tissue Array.
- 3-D model elaboration as mesh polygonal structure using a specific algorithm
- model rendering, in which we provide the rough wire frame model with a texture characterized by a specific color, a particular shininess and transparency degree.

Such elaborated models were exported in DXF format, and further manipulated by a second software, Amira 3.1.1 (TGS, Template Graphics Software), which allowed to generate cross-sections at various cut planes, overcoming in such way the usual limits imposed by conventional histological examination; these virtual sections were visualized as fixed pictures or progressively in motion.

## Results

A total of 13 3-D histopathological models were created.

Concerning methodological aspects, Tissue Array technology showed to be easy to perform,



**Fig. 2** Schematic representation of the use of Tissue Array to determine the distance between tissue sections. A perpendicular array (x) and a leaning one (y) were introduced by attaching the tissue block to be studied over an inclined holder of a given angle. The distance between sections (t) was related to the distance, in different sections ( $a_1$  and  $a_2$ ), between array x and array y, as well as to the angle ( $\alpha$ ) of the leaning array, as follows:  $[t = (a_2 - a_1) \tan \alpha]$ .

applicable to archive material, and fast. In the here-in proposed 3-D modelling procedure, as well as for most of other methodologies reported, time limits were mostly due to block sectioning, while the Tissue Array procedure required only ten to fifteen additional minutes for each block. Since in some instances, even depending on the type of tissue analyzed, one or more cores of the fiducial markers may be lost in sectioning processes, the use of 3 to 5 tissue markers for each recipient sample block gave the best reproducibility. Anthracotic lymph node, cerebral cortex or skeletal muscle tissues did not show significant differences as source of fiducial marker and all could easily be recognized inside the sections of the recipient tissue block. In the 3-D modelling step the use of tissue array as fiducial markers proved to be an easy device in image overlap of the digital pictures of serial sections, with highly reproducible model elaboration when either of the two softwares was employed. Another crucial point is represented by the possibility to calculate the distance between the serial sections. Although, it can be calculated indirectly knowing the thickness and number of sections in the interval, we demonstrated that it can be easily obtained by embedding the TA reference markers at different angles using proper formulas (see Fig. 2 and 3).

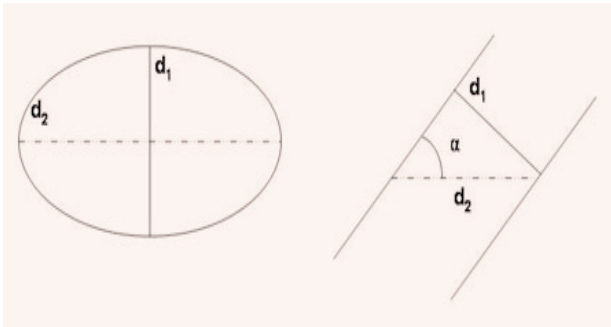
With respect to 3-D models created, 5 fetal hearts were reconstructed. In 4 of these, a normal anatomy, appropriate for gestational age, was



observed by both macroscopic examination, histological morphology and 3-D models. In the fifth case, despite an apparently normal macroscopic and histological structure, 3-D modelling revealed the presence of a ventricular septal defect, just below the atrio-ventricular valves (Fig. 4).

With reference to tumor samples, 3-D models comprised 5 cases of breast carcinoma, which consisted of 3 cases of ductal carcinoma in situ (DCIS), including 1 case of low grade and 2 cases of high grade carcinomas, 1 case of lobular carcinoma in situ (LCIS) and 1 case of tubular carcinoma. 3-D reconstruction was able to determine the ductal arborisation pattern in all three histological types of carcinoma, showing the fine network of ductal and ductular structures interconnecting each other (Fig. 5).

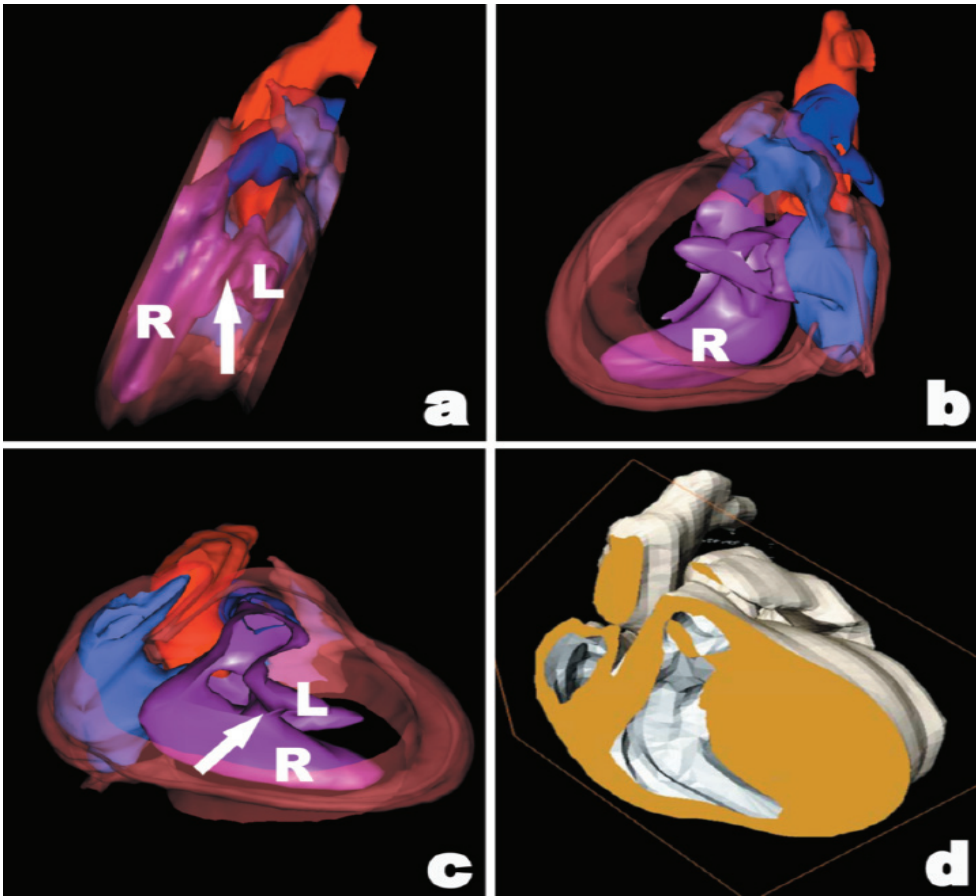
In two of the three examples of thyroid carcinoma, one case of papillary carcinoma and one case of follicular carcinoma, the 3-D pattern of vascular invasion was investigated. Serial sectioning and 3-D alignment showed that vascular invasion within or outside the tumor capsule initiate as capsular penetration by tumor cells through (probably pre-

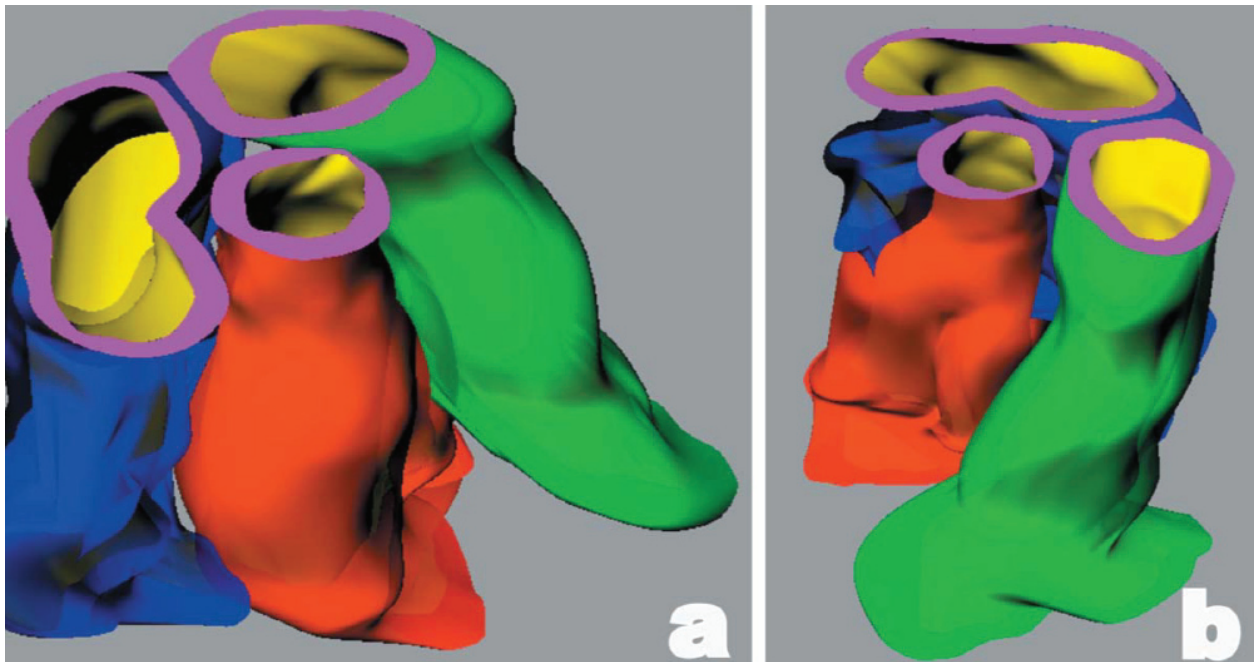


**Fig. 3** Schematic representation of the possibility to calculate the angle (see Fig. 2) by using the formula  $\alpha = \arcsin(d_1/d_2)$ , where  $d_1$  and  $d_2$  are the diameters of the section of the leaning array  $y$ .

existing) orthogonal vascular structures of the tumor capsule, then running tangentially within the capsule thickness (Fig. 6). In the third case of thyroid carcinoma, a follicular carcinoma, the architecture of vascular structures in the periphery of the tumor and the possible connections to the tumor capsule were determined.

**Fig. 4** 3-D model reconstruction of a fetal heart, bearing a ventricular defect (arrows in projections a and c). Planar sections may also be obtained (d). L: left ventricle; R: right ventricle.





**Fig. 5** 3-D representation of adjacent ductal structures in a case of high grade DCIS. The 3 ducts appear rigid and show no interconnections one to the other.

## Discussion

Since the middle of the nineteenth century, as the knowledge on the microscopic structure of tissues rapidly increased, several investigators aimed to define the spatial 3-D structure corresponding to the conventional histological view. Several different methods were subsequently created [2, 10, 11], but failed to become popular since their common limits in terms of time consume and restricted applicability to specifically processed tissue samples. One of the major trouble is still represented by the correct alignment of serial tissue sections, in order to prevent model rendering errors due to unrecognized spatial details of the object under investigation. Recently, digitalization of histological images partially overcame the problem, but a simple and reliable method to insert reference markers in the tissue specimens and preventing 3-D model errors due to misalignment biases is still needed.

In the present study, we focused on the possible application of the novel Tissue Array methodology in incorporating fiducial tissue markers to be used in 3-D reconstruction procedures. Tissue Array is a widespread methodology, recently described, which

allows to selectively capture tissue cores (0,6 to 2 mm in size) from donor paraffin blocks so as to assemble recipient paraffin blocks representative of up to hundreds of different tissue donor samples [12]. The main advantage of such a method is represented by sensitive decrease of cost and time consume when applying alternative techniques (i.e. immunohistochemistry or in situ hybridization) on large series of tissue samples [13]. To our knowledge, a possible applicability of Tissue Array in 3-D modelling procedures has not been reported to date. In our hands, due to its feasibility in archive (not otherwise specifically processed) paraffin material, as well as its fast, easy and reproducible procedure, Tissue Arrays represent a prompt improvement in 3-D reconstruction, and imply open perspectives in the diffusion of 3-D studies in both normal and pathological tissues.

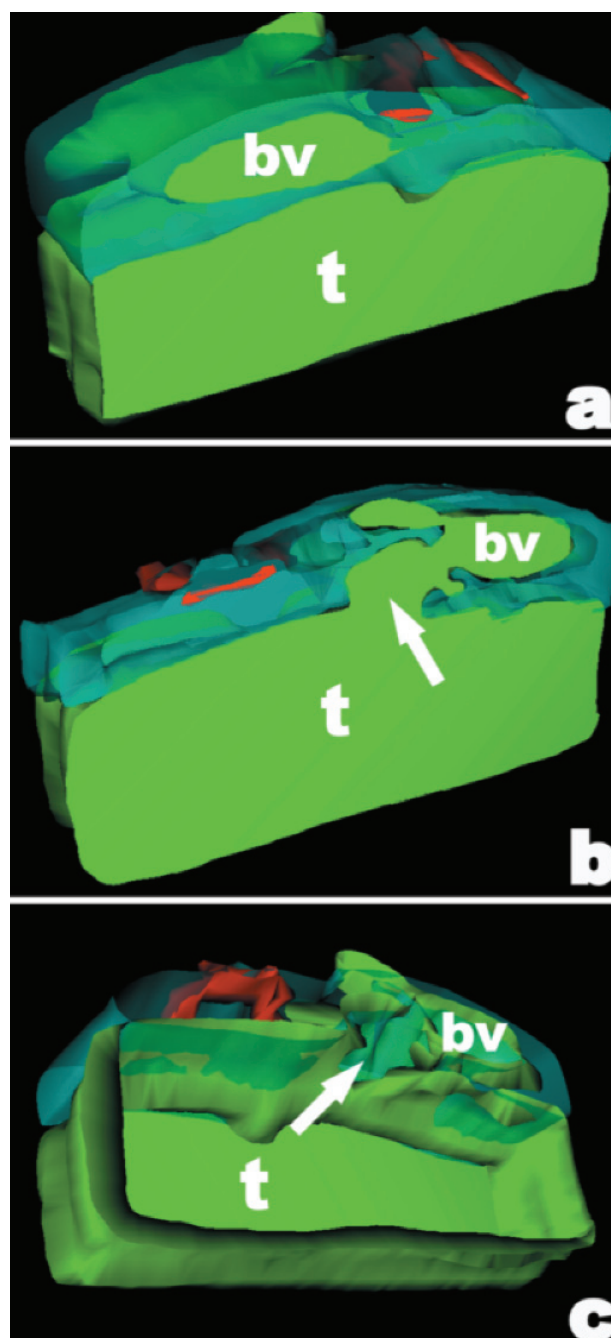
We are here presenting 13 examples of the feasibility of the proposed procedure in order to reach 3-D reconstruction. The results and interest of such reconstructions will be expanded in future study and will only shortly be outlined here. One of the most important application of 3-D reconstruction in pathology is represented by the analysis of tiny structures not easy to evaluate by conventional

macroscopic examination, such as the case of fetal cardiopathology, where the dimensional limits of the organs under investigation in most cases prevent accurate diagnosis of the presence of malformations. In our preliminary study, the 3-D approach we applied showed to be more sensitive than macroscopic and histological examination to determine the presence of a fetal heart malformation. Thus, 3-D reconstruction represents an useful additional diagnostic tool in this specific field, where the increasing progress in the pre-natal ultrasonographic diagnosis of cardiac malformations raise challenging diagnostic problems for the pathologists related to the fine examination of minute cardiovascular apparatuses [14].

In tumor pathology, although 3-D model elaboration may not be considered a practical approach in the diagnostic routine practice, it may offer interesting perspective in the insight of histopathology of tumors. As an example of this, our results on the 3-D reconstruction of vascular invasion in thyroid tumors, could have important implications in redefining the criteria of malignancy in thyroid nodules. In fact, according to the 3-D models we obtained, it seems to be feasible that capsular and vascular invasions in thyroid follicular neoplasms represent two morphological features of the same phenomenon. Our data are partially consistent with other studies on the spatial distribution of peritumoral blood vessels in thyroid nodules [15, 16].

As an other example, we applied our 3-D approach to define the ductal and ductular architecture of *in situ* breast carcinomas, which has been a matter of long debate in the past years [17, 18]. We observed in low grade DCIS a discontinuous spatial distribution of tumor tissue within the ductal arborisation, while in the case of high grade DCIS the tumor was homogeneously distributed along ductal structures, without interposition of normal epithelium, possibly as the result of alternative mechanisms of tumor spread and multifocality in *in situ* breast carcinomas.

In conclusion, we present a distinctive application of Tissue Array technology as the source of fiducial markers for a 3-D reconstruction procedure based on alignment of serial histological sections. The methodology proposed is easy to perform, accurate and reproducible, and could be considered an additional tool for pathologists in both research purposes and diagnostic applications in specific fields of interest.



**Fig. 6** 3-D demonstration of vascular invasion in follicular thyroid carcinoma. Tumor tissue (t) permeate the capsular thickness and protrude into a peritumoral blood vessel (bv) through a capsular break (arrows, in b and c).

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